COMPARING MODELS FOR VESICANT RESPONSES IN SKIN CELLS

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ABSTRACT

Vesicant challenges have been delivered to NHEK (normal human epidermal keratinocyes) and to artificial human epidermal tissues. Confluent NHEK, grown on plastic surfaces or gel-coated microporous membranes of Millicell CM® inserts, were challenged with vesicants diluted in cell culture medium. Testskin® was provided on agarose nutrient gel as a cornified wafer of sufficient diameter to receive vesicant vapor from cups normally used to challenge animal skin. Stratum corneum of pre-production EpiDerm® (PreEpiD) specimens were challenged with vesicant vapor from cups suspended inside of Millicells. Inverted phase contrast microscopy of NHEK on plastic revealed dose-related vesicant effects that could facilitate screening of antivesicants. Scanning electron microscopy (SEM) revealed vesicant effects in two distinctly different populations of NHEK on gel-coated inserts. SEM and transmission electron microscopy (TEM) of Testskin and PreEpiD disclosed structural differences between these models that became amplified in vesicant-challenged specimens. PreEpiD shows more promise than Testskin for screening of antivesicant topical skin protectants. However, both epidermal models lack the basal lamina that is needed for advanced antivesicant testing.

INTRODUCTION

Models of artificial human epidermis were used during studies to determine feasibility of using spectrofluometric methods[1] to measure effects of vesicant compounds *in vitro*[2]. One model was Testskin, also called Living Skin Equivalent® (LSE®). This was fabricated by Organogenesis, Inc. (Cambridge, MA) from Living Dermal Equivalent® (LDE®) seeded with NHEK and fed from below to form stratum corneum. This model is no longer being marketed.

Three different epidermal models are made by seeding NHEK onto the microporous membranes of Millicell CM inserts (Millipore Corporation, Bedford, MA). The cells are fed from below via the membrane in each case. Two of the models require coating of the Millicell membrane with a cross-linked collagen gel. These are the non-cornified Millicell model[3] and the human epidermal model[4]. The Millipore Corporation has patented this methodology and licensed it to the MatTek Corporation (Ashland, MA), which markets EpiDerm. The methods used to support EpiDerm in Millicells are proprietary.

The least complicated model consists of NHEK attached to wells of plastic cell culture plates. Such wells also hold Millicells.

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OBJECTIVES

The initial objective of this study was to identify the best skin cell models for testing of antivesicant compounds in a bottom-reading spectrofluorometer. A secondary objective was to determine whether such models could be optimized for test purposes.

METHODS AND MATERIALS

Millipore's Cytofluor 2300® spectrofluorometer reads dye probe signals[2] that have been generated within NHEK damaged by vesicant compounds[1]. This instrument reads through plastic culture plates[3,4]. NHEK under aqueous medium are adherent to the flat-bottoms of 24-well polystyrene culture plates (Falcon® 3847, Becton Dickinson and Company, Oxnard, CA). Alternately, wells of plates may contain medium and Millicell CM inserts or Testskin. The coated membrane (Millicell) or LDE (Testskin) that supports the NHEK of the model must be transparent to light signals and retain much less dye than the NHEK[1]. Each of the models in this comparison has been found to be usable with the Cytofluor 2300 by methods described elsewhere[1].

Initial vesicant challenges of NHEK (Clonetics, San Diego, CA) on gel-coated Millicells were made with a non-surety vesicating agent, 2-chloroethyl ethyl sulfide (CEES), in anhydrous ethanol. Serial dilutions were made to 0.8, 8.0 and 80 mM in cell culture medium[1]. Sulfur mustard (HD), also in ethanol, was diluted to final concentrations of 0.1, 1.0, 5.0, 10, 20 and 100 mM in medium over cell culture plates. Vapor challenges of 4- and 8- min duration were made via animal exposure vapor cups[1] over Testskin. Similar HD vapor challenges of PreEpiD were made with vapor cups of smaller diameter that were suspended within the PreEpiD Millicells.

Inverted phase contrast microscopy of NHEK monolayers and electron microscopy of other epidermal models were used to observe (1) control monolayer uniformity, (2) ease of interpreting vesicant responses and (3) relevance for interpretation of human responses to vesicant compounds. Micrographs display features of epidermal models that bear on their possible use for initial screening, intermediate comparisons or advanced optimization of antivesicants.

OBSERVATIONS

The results are summarized in Figures 1-4. These results fall under the following headings:

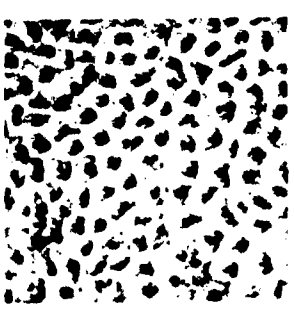
A. MODELS FOR USE TO REPRESENT BASAL CELL RESPONSES TO HD

Fig. 1: Skin Cell Monolayer...NHEK confluent on plastic plate.
Fig. 2: Cell Layers in Millicell...NHEK on gel-coated insert, confluent, stratified, non-cornified.

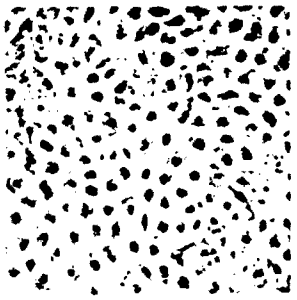
B. MODELS FOR TESTING OF SKIN PROTECTANTS AND ANTIVESICANTS:

Fig. 3: PreEpiD (pre-production EpiDerm)...NHEK on Millicell CM microporous membranes, differentiated, cornified.
Fig. 4a: Testskin...NHEK on LDE, differentiated, cornified.
Fig. 4b: Human Epidermal Model...NHEK stratified with basal lamina on gel-coated Millicell insert, differentiated, cornified.

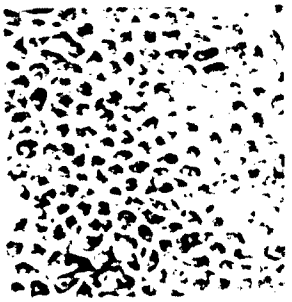
FIGURE 1. HUMAN SKIN CELL MONOLAYER MODEL, WITH VARIATIONS



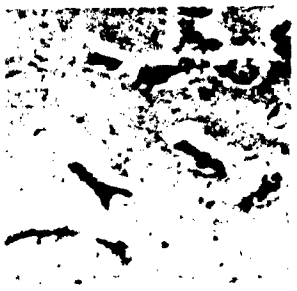
A. NHEK controls on plate w/ intercell. spacing normal



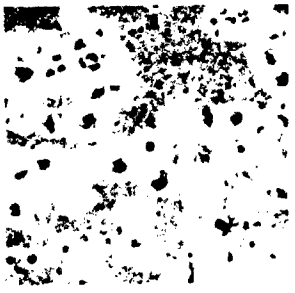
B. Widened intercell. spaces, 4 hrs after 0.01 mM HD in medium



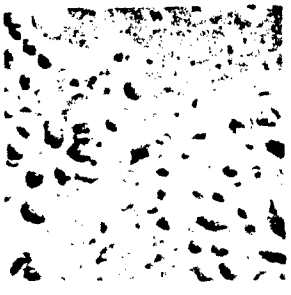
C. Very wide spaces & "lifting", 4 hrs after 0.1 mM HD



D. Normal endothelial cells with spatulate "endo" shape on plate



E. Endo "Rounding up" & lifting from plate w/ 20.0 mM HD + 4 hrs



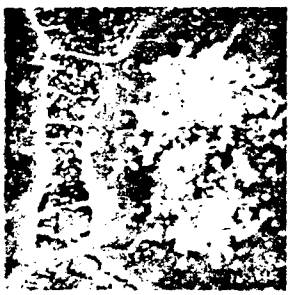
F. NHEK Rounding up, & "lifting" 4 hrs after 20.0 mM HD

G. Subconfluent NHEK normal monolayer on plastic plate

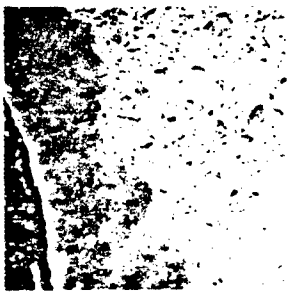
H. Subconfluent NHEK non-monolayer showing differentiated island

I. Confluent NHEK differentiating on plastic plate

FIGURE 2. SEM OF NHEK ON GEL MILLICELL® MODEL, WITH VARIATIONS

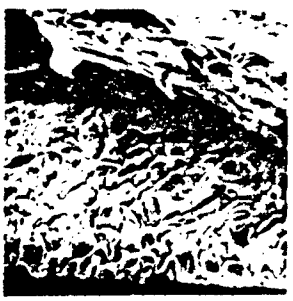


A. Squamous cells in top layer of NHEK on gel-coat of Millicell



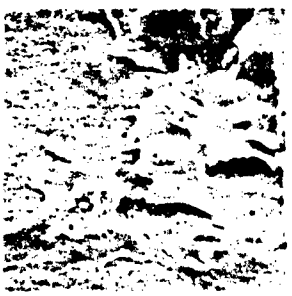
B. Membrane necrosis of squamous cell at +24 hr w/ 80 mM CEES

C. Delamination & erosion at 24 hrs after 80mM CEES

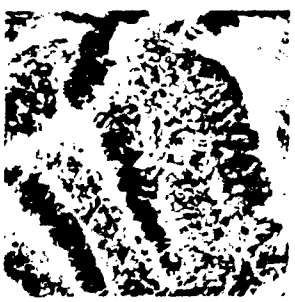


D. Basal cells on gel membrane w/ squamous cells peeled upward

E. Squamous cells on top of basal cells of thin multilayer

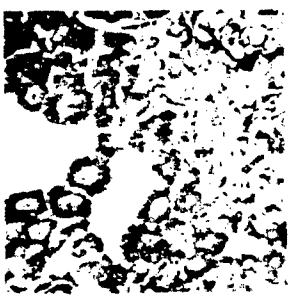


F. Subconfluent new squamous cells on gel-coating (left)



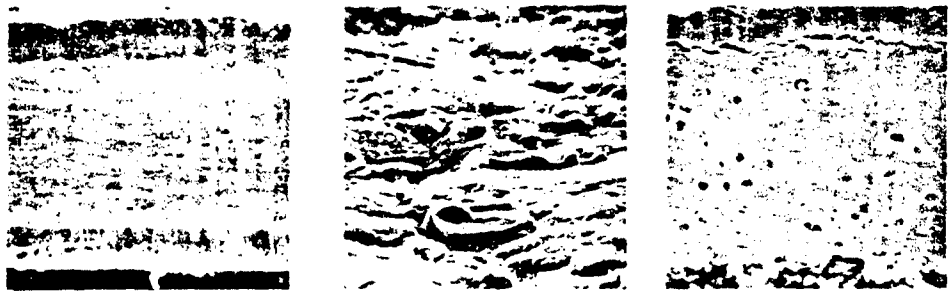
G. Basal cells on gel, squamous cells peeled off (see cut D, above)

H. Proliferation of basal cells on gel-membrane of insert



I. Diverse forms of NHEK on gel (left) of Millicell CM

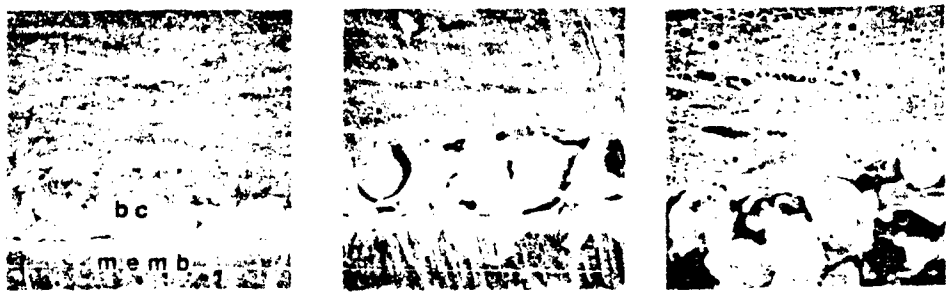
FIGURE 3. PRE-PRODUCTION EPIDERM® MODEL (PreEpiD MODEL)



A. PreEpiD control, enlarged in cut D

B. PreEpiD keratin 12 hr after HD vapor

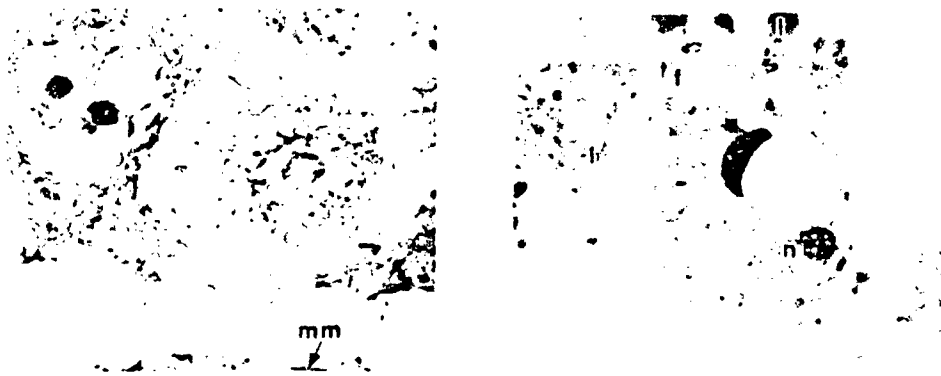
C. PreEpiD spinosum at 24 hr; HD vapor



D. PreEpiD basal cells (bc) & membrane (memb)

E. bc contracting 12 hr after HD vapor

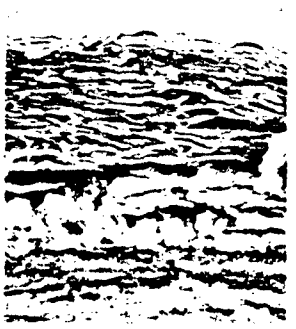
F. PreEpiD bc at 24 hrs after HD vapor



G. TEM of an PreEpiD Basement Membrane Zone. Contraction & separation of bc from mm (microporous membrane)

H. TEM; PreEpiD, 8 min HD vapor + 24 h. Karyorrhectic nuclei (n), lipid incl.(l) & coagulated bc tonofilaments (tf). 9000X.

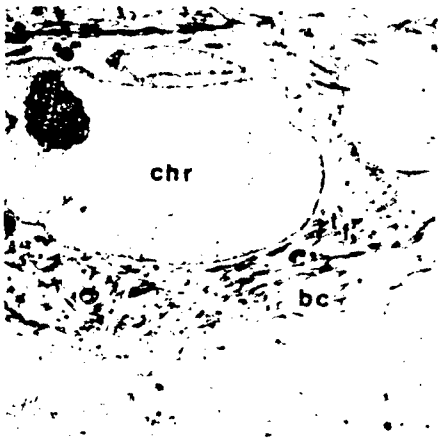
FIGURE 4. MICROGRAPHS OF EPIDERMAL MODEL BASAL MEMBRANE ZONES



A. Testskin control showing disordered spinosum over basal cells over LDE.

B. Testskin control with displacement of basal cells and torn spinosal strata.

C. Testskin specimen showing effects (of 8 min of HD vapor + 24 hrs) like control



D. TEM of Testskin Basement Membrane Zone with structural components and hemidesmosomes absent. Basal cells (bc) show typical appearance of chromatin (chr) and tonofilaments (tf)

E. TEM of Human Epidermal Model differentiated for 10-14 days w/ increased extracellular Ca^{+2}. Electron-dense lamina (L) is indicated at interface of bc and gel-membrane over insert.

DISCUSSION

Figure 1, cuts A-C, suggests that the monolayer model may provide an *in vitro* analogue of the epidermal cell separation that is a prominent feature[4] of HD effects within human and animal epidermis. Cuts D-F provide the basis for one hypothesis that may relate to both *in vitro* and *in vivo* cases. During attempts to propagate normal umbilical vein endothelial cells (NUVEC, a gift of Clonetics Corporation) it was observed that these cells are very readily detached from cell culture flasks with a trypsin/EDTA solution (CC5013, Clonetics). An appearance like that of cut E was observed minutes sooner than with comparably treated NHEK (see cut F). It is hypothesized that methods for the study of trypsin and cellular adhesion mechanisms may apply to investigation of the HD effects in skin cells.

Figure 1, cuts G-I, provides visible evidence of variables that need to be controlled for most efficient use of the monolayer model in screening of antivesicant compounds. Cut A and cut G reveal considerable differences in the morphologies of individual NHEK in these well-behaved monolayer preparations. Cuts H and I illustrate departures from monolayer characteristics. The differentiation of NHEK on plastic is thought to be suppressed with lateral contact inhibition and low Ca^{++} in the culture medium[5]. Basal cells that divide readily are considered more sensitive to vesicants than the more differentiated epithelial cells[4]. Therefore, cuts G-I warn us that an optimized monolayer model should be uniformly proliferative and have equivalent numbers of cells for reliable use with fluorescent dyes[6].

Figure 2, cuts A-I, reveals much greater tendencies for NHEK differentiation and model diversity than was revealed in Figure 1. Fluorescent dye studies with this model were promising,[7] but results of the tests indicate that the best data were obtained from parallel comparisons involving a single lot of cells. Studies with different lots did confirm dye response patterns, but fluorescence levels were variable[8]. Cuts A-C suggest that squamous cells protect basal cells from calculated vesicant concentrations. Cuts C-E show the squamous cells forming multiple layers that overlie basal cells. Basal cells may be similarly insulated by the underlying gel-coat from direct contact with vesicant in the medium. Variations in squamous cell layers and/or gel-coating affect fluorescent dye readings in the Cytofluor 2300[9].

Figure 2, cuts F-I, illustrates basal cell variation in the Millicell model. Cuts D and G depict an apparently uniform layer of rectilinear basal cells applied to the underlying gel-membrane. However, cuts F, H and I indicate potential variability of basal cell layers in other Millicells. Cut F suggests a senescent cell population that rapidly becomes squamous although it is in direct contact with the gel-membrane. Cut H has the appearance of vital and rapidly proliferating NHEK. Cut I shows a mixture of NHEK clones that represent differing degrees of senescence. NHEK vary in viability from lot to lot and all die after 4-8 passages[6].

Figure 3, cut A, shows a small section from one of many SEM micrographs of normal control PreEpiD. The original print included a view of stratum corneum that resembled sections from human skin.

Cut A shows the dense, uniform structure typical of the spinosum layers in other PreEpiD specimens. Cuts A and E both show well-formed and densely packed basal cells that are partially attached to the underlying microporous membrane of a Millicell CM insert. This membrane shows no evidence of any gel-coating. Neither the microporous membrane nor the basal cells have any attachment suggesting basement membrane structure of skin; however, overlying structures do resemble human skin.

Figure 3, cut B, provides a somewhat eroded indication of the stratum corneum appearance in SEM micrographs of control specimens. An 8-minute HD vapor challenge appears, after 12 hrs, to show a lesser degree of superficial damage (in cut B) than is seen at 24 hours after an 80 mM CEES exposure (Figure 2, cuts B and C). This apparent difference may be misleading. It is possible that the observed CEES damage may have resulted from liquid droplets that separated from medium saturated with CEES[3].

Figure 3, cut C, reveals substantial effects of HD vapor in the spinosum-like layers of PreEpiD. Cut C is an SEM view of HD vapor effects after an 8-minute exposure about 24 hours before fixation of the specimen. Cuts D and G provide control standards for evaluation of HD vapor effects on basal cells, as seen in cuts E, F and H. Cut E and other SEM micrographs (not shown) suggest that basal cells are damaged but still partially attached to the microporous membrane at 12 hours after the specified HD exposure. Cuts C, F and H show that this vapor challenge results in basal cell damage severe enough (after 24 hours) to represent an *in vitro* analogue of blister formation *in vivo*. Earlier results with HD vapor exposures of Testskin suggest that such separation may take place *in vitro* in less than 12 hours[7]. These and other TEM observations suggest that some human epidermal equivalent models are likely to match specifications for HD pathology requirements[4].

As noted above, Testskin is no longer available (although the HSE and HDE methodology are presumably used in Graftskin, the new Organogenesis product). Figure 4, cuts A-C, suggest that problems with uniformity of Testskin specimens may have driven the change to a less demanding application. These and other TEM micrographs (not shown) reveal a general tendency of Testskin to contain columns of cells of differing packing densities. Although NHEK used in the fabrication of Testskin may differ from the Clonetics variety, the observations from Figure 2 suggest that clonal variations could account for some of the observed Testskin diversity. Variations in LDE may have compounded the variability. The appreciably smaller diameter and the shape preserving properties of the Millicell unit may partially explain why PreEpiD seems to have overcome the same problems. Tissue geometries and cell shapes are important factors in epidermal differentiation[5].

The absence of a basement membrane in PreEpiD is illustrated in Figure 3, cuts A, D and E. Figure 4, cut E, shows the close apposition of Testskin epidermis to underlying HDE. Cut E also shows that apposition of epidermal and dermal equivalents does not necessarily lead to basal membrane formation. Figure 4, cut F, is evidence of basal lamina formation after all of the MatTek-licensed technology is brought to bear. Figure 3 provides evidence that the fabrication of the PreEpiD model does not include use of the gel- coating methodology that appears to account for the basal lamina shown in Figure 4, cut F.

CONCLUSIONS

1. The less expensive NHEK monolayer model shows separation of cells that is an advantage over the non-cornified Millicell model for initial screening of candidate antivesicant compounds.

2. The uniformity and optimal differentiation state of future NHEK monolayer model specimens must be improved if this model is to be used efficiently for screening purposes.

3. PreEpiD (pre-production EpiDerm) may be suitable for screening of topical skin protectants and decontaminants.

4. A model of human epidermis must contain a functional and structural equivalent of basement membrane if it is to be used for advanced screening of antivesicants intended to limit blistering.

ACKNOWLEDGEMENTS

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